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(54) **hG-CSF and method for obtaining the same**
hG-CSF und Verfahren zu seiner Herstellung
hG-CSF et méthode de préparation

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Description

[0001] The present invention relates to a colony stimulating factor (hereunder referred to as G-CSF) that has the ability to promote the differentiation and proliferation of bone marrow cells. More particularly, the invention relates to a novel G-CSF that has the ability to promote the differentiation and proliferation of human bone marrow cells to neutrophiles (such particular CSF may hereunder sometimes be referred to as human G-CSF) and a method for obtaining the same.

[0002] The G-CSF in accordance with the present invention has the potential for use not only as a curative for leukopenia but also as a reagent for clinical testing and research studies.

[0003] Colony stimulating factors (CSFs) are responsible for the development of myeloid and erythroid cells. They act on animal bone marrow cells so as to promote their differentiation and proliferation to macrophages or granulocytes. Several types of CSFs have been reported. For example, Stanley, E. R. et al. reported that they purified from the urine of healthy adults a CSF that was composed of a glycoprotein with a molecular weight of 45,000 and which exhibited a colony stimulating activity on mouse bone marrow cells but not on human bone marrow cells (Fed. Proc., *35* (1975), 2272-2278). Burgess, A. W. et al. reported that a CSF effective in humans was partially purified from human placenta (Blood, *49* (1977), 573-583 and ibid, *54* (1979), 614-627). Shah, R. G. et al. reported the partial purification of a similar CSF from monocytes in human peripheral blood and PHA-stimulated lymphocytes (Blood, *50* (1977), 811). Fojo S. S. et al. reported the partial purification of a similar CSF from the supernatant of a culture of human lung cells (Biochemistry, *17* (1978), 3109-3116). All of these CSFs are glycoproteins having molecular weights in the range of 25,000 to 41,000 and they act directly on non-adherent human bone marrow cells to form colonies of neutrophiles, macrophages and eosinophiles. However, because of limitations on the available sources, completely purified CSFs have not yet been obtained. In addition to these CSFs recovered from normal human tissues, some kinds of human tumor cells have recently been reported to have the capability of CSF production. For instance, Asano, S. et al reported the recovery of CSF from lung cancer cells transplanted into nude mice (Blood, *49* (1977), 845-852). Okabe, T. et al. reported CSF production by a cell line of mandibular squamous cell carcinoma and thyroid gland cancer cells (Cancer Res., *38* (1978), 3910-3917; JNCI, *69* (1982), 1235-1243; and J. Cell Physiol., *110* (1982), 43-49). Wu, M. C. et al. reported CSF recovery from a pancreas cancer cell line (J. Biol. Chem., *254* (1979), 6226-6228; J. Clin. Invest., *65* (1980), 772-775). Dispersio, J. F. et al. reported that they recovered CSF from a GCT Cell line established from patients with malignant histiocytoma (Blood, *51* (1978), 1068: and Blood, *56* (1980), 717-727). Golde, D. W. et al. reported the recovery of CSF from an MO cell line established from patients with hairy cell leukemia (Blood, *52* (1978), 1068-1072; and Blood, *57* (1981), 13-21). The CSFs, which are effective on human bone marrow cells are glycoproteins having molecular weights ranging from 27,000 to 34,000 and isoelectric points (pI) of 4.5-5.7. The CSF obtained from the supernatant of a culture of the GCT cell line has been purified to a specific activity of 1.12×10^6 U/mg. The specific activity of the CSF obtained from the supernatant of a culture of the MO cell line has been increased to 3.5×10^6 U/mg. However, none of these CSFs have been purified completely. In addition CSF which has the ability to specifically promote the differentiation and proliferation of human bone marrow cells to granulocytes has not been reported to date.

[0004] The present inventors have suceeded in establishing a cell line from tumor cells of a patient. This cell line is able to produce G-CSF. It has been deposited with Collection Nationale de Cultures de Microorganismes, (C.N.C.M.) Pasteur Institute, France on July 11, 1984 under the Deposit Number I-315.

[0005] According to the present invention this cell line I-315 is cultured in vitro, and from the supernatant of the culture a highly pure G-CSF which exhibits a human neutrophilic colony stimulating activity, and which had a molecular weight of about 18,000 (as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)) and a specific activity of 3.94×10^7 U/mg in the human bone marrow cell assay or higher can be isolated. In one aspect, the present invention relates to a G-CSF having the following physicochemical properties which are not shown in literature and makes this G-CSF a novel substance.

i) Molecular weight:
19,000±1,000 as determined by sodium dodecylsulfatepolyacrylamide gel electrophoresis;
ii) Isoelectric point:
Having at least one of the three isoelectric points, A, B and C, shown in Table 1:

TABLE 1

Isoelectric point (pI)		
	In the presence of 4M urea	In the absence of urea
A	5.7±0.1	5.5±0.1
B	6.0±0.1	5.8±0.1
C	6.3±0.1	6.1±0.1

iii) UV absorption:
Maximum absorption at 280 nm and minimum absorption at 250 nm;
iv) The N-terminal 21 amino acids are

(5) (10) (17) (20)
H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser- Phe-Leu-Leu-Lys-X-Leu-Glu-X-Val-;

wherein X represents a naturally occurring unidentified amino acid residue.

[0006] The G-CSF of the present invention has the ability of promoting the differentiation and proliferation of human bone marrow cells to neutrophilic granulocytes but not to granulocyte-macrophages and not to eosinophils in the human bone marrow cell assay at days 7, 10 and 14 of the incubation.

[0007] In another aspect, the present invention relates to a method for obtaining a G-CSF which comprises culturing a cell line having the ability to produce human G-CSF having the physicochemical properties shown above, subjecting the supernatant of the culture to steps (1) to (3) indicated below, and optionally subjecting the resulting fractions to either step (4) or (5):

step (1) subjecting the supernatant of the culture to gel filtration using a gel having an effective fraction range of 5,000-70,000 daltons, and recovering fractions having the neutrophile-dominant colony stimulating activity (CSA);
step (2) adsorbing the recovered fractions onto a carrier for reverse-phase high-performance liquid chromatography and performing elution by the density gradient technique with a mixture of water and an organic solvent so as to recover fractions having the neutrophile-dominant CSA;
step (3) subjecting the so recovered fractions to high-performance molecular sieve chromatography so as to recover fractions having the neutrophile-dominant CSA;
step (4) subjecting the so recovered fractions to isoelectric point electrophoresis (isoelectric focusing) so as to recover fractions having the neutrophile-dominant CSA; or
step (5) subjecting the fractions recovered in step (3) to the step of removing sialic acid so as to recover fractions having the neutrophile-dominant CSA.

Figure 1 shows the results of SDS-PAGE performed on the G-CSF of the present invention which is indicated by the dot in the graph;
Figure 2 shows the results of isoelectric electrophoresis (isoelectric focusing) performed on the G-CSF of the present invention in the presence of 4M urea;
Figure 3 is a UV absorption spectrum chart for the G-CSF of the present invention;
Figure 4 shows the proliferation profile of I-315; and
Figure 5 is another graph showing the results of SDS-PAGE performed on the G-CSF of the present invention which is indicated by the dot in the graph.

[0008] An outline of the method for obtaining the G-CSF of the present invention is hereunder described with reference to I-315.

[0009] A sample of I-315 is suspended in an F-10 culture solution (Ham R.G. Exp. Cell Res. *29* (1963), 515) containing 10% of fetal calf serum (FCS) and subjected to rotary incubation in a glass roller bottle at a constant rate. When the inner wall of the roller bottle has been completely covered with CHU-1; the culture solution is replaced by FCS-free RPMI 1640, which is subjected to incubation for 4 days. To the recovered supernatant of the culture, FCS-containing F-

10 culture solution is added again, and incubation is conducted for 3 days. The culture solution is again replaced by FCS-free RPMI 1640, and after incubation for 4 days, the supernatant of the culture is recovered. In accordance with this schedule, cycles of "incubation and recovery of the supernatant of serum-free culture" are repeated to obtain the final supernatant of culture of I-315. The supernatant thus obtained is subjected to ultrafiltration to obtain an approximately 1,000-2,000-fold concentrate, which is subjected to gel filtration to recover fractions having the neutrophile-dominant CSA. The fractions are subjected to repeated purification by high-performance liquid chromatography, thereby recovering portions having the neutrophile-dominant CSA. The portions are subsequently freeze-dried.

[0010] For the purposes of the present invention, CSA was determined by one of the following two methods.

(a) Using human bone marrow cells:

Incubation on a monolayered soft agar culture was conducted in accordance with the method of Bradley, T. R. and Metcalf, D. (Aust. J. Exp. Biol. Med., Sci., *44* (1966), 287-300). Fetal calf serum (FCS, 0.2 ml), a test sample (0.1 ml), a suspension of non-adherent human bone marrow cells (0.1 ml, containing $1\text{-}2\times10^5$ nuclear cells), modified McCoy's 5A culture solution (0.2 ml) and 0.75% agar containing modified McCoy's 5A culture solution (0.4 ml) were mixed and poured into a plastic dish (35 mm$^{\varnothing}$) for tissue culture. Incubation was conducted at 37°C and 100% humidity with 5% CO_2/95% air. The number of colonies formed (one colony consisting of 50 or more cells) was counted after ten days of incubation, and the activity capable of forming one colony was taken as one unit of CSA.

(b) Using mouse bone marrow cells:

Horse serum (0.4 ml), a test sample (0.1 ml), a suspension of C3H/He (female) mouse bone marrow cells (0.1 ml, containing $0.5\text{-}1\times10^5$ nuclear cells) and 0.75% agar containing modified McCoy's 5A culture solution (0.4 ml) were mixed and poured into a plastic dish (35 mm$^{\varnothing}$) for tissue culture. Incubation was conducted at 37°C and 100% humidity with 5% CO_2/95% air for 5 days. The colonies formed were counted (one colony consisting of 50 or more cells), and the activity capable of forming one colony was taken as one unit of CSA.

The modified McCoy's 5A culture solution used in each of the methods (a) and (b) and the suspension of non-adherent human bone marrow cells used in method (a) were precared by the following procedures.

Modified McCoy's 5A culture solution

[0011] Twelve grams of McCoy's 5A culture solution (product of Gibco Co.), 2.55 g of MEM amino acid/vitamin medium (product of Nissui Seiyaku Co., Ltd.), 2.18 g of sodium bicarbonate and 5,000 units of potassium penicillin G were dissolved in 500 ml of twice-distilled water, and the solution was sterilized by passage through a Millipore® filter (0.22 μm).

Suspension of non-adherent human bone marrow cells

[0012] Bone marrow cells obtained from a healthy adult by sternal puncture were diluted 5-fold with RPMI 1640 culture solution. The dilution was placed over Ficol-Paque solution (product of Pharmacia Fine Chemicals), and the mixture was centrifuged at 400xg and 25°C for 30 minutes to recover the interfacial cell layer (specific gravity <1.077). The layer was washed with RPMI 1640 solution and the cell number was adjusted to a concentration of 5×10^6 cells/ml with RPMI 1640 culture solution containing 20% of FCS. The solution was poured into a 25-cm^2 plastic flask for tissue culture. After incubation in a CO_2 incubator for 30 minutes, non-adherent cells were recovered from the supernatant and put into a plastic flask (25 cm^2). After a 2.5-hr incubation, non-adherent cells were collected from the supernatant.

[0013] When the CSF of the present invention was allowed to act on mouse bone marrow cells and human bone marrow cells as will be shown later in Example 6, stimulated formation of granulocytes colonies was observed. This clearly indicates that the G-CSF of the present invention is of the type that promotes the differentiation and proliferation of bone marrow cells to granulocytes.

[0014] The method of establishing cell line I-315 is as follows:

(i) Tumor:

Pieces of tumor tissue from a patient with oral cancer accompanying a remarkable increase in the number of neutrophiles was transplanted into a nu/nu mouse. About 10 days after the transplantation, a remarkable increase in the size of the tumor and the number of neutrophiles was observed. 12 days from the transplantation, the tumor was aseptically removed from the mouse and divided into small pieces (1-2 mm^3), which were subjected to the following incubations.

(ii) Primary culture:

Ten to fifteen tumor pieces were put into a 50-ml plastic centrifuge tube. After addition of 5 ml of a trypsin solution (0.25% trypsin and 0.02% EDTA), the mixture was shaken in a warm bath (37°C) for 10 minutes. The supernatant was discarded and 5 ml of a tryspin solution having the same composition as used above were added and

the trypsin digestion was performed under agitation at 37°C for 15 minutes. A cell suspension was recovered and mixed with 1 ml of FCS so as to prevent the action of trypsin. The cell suspension was stored in an ice bath.

The same procedure was repeated to recover a cell suspension which was combined with the previously obtained suspension and centrifuged at 1,500 rpm (400xg) for 10 minutes to obtain cell pellets. The pellets were washed twice with Medium F-10 [Ham. R. G., Exp. Cell Res. 29 (1963), 515] culture solution containing 10% FCS and transferred to a plastic incubation flask (25 cm^2) to give a concentration of 5×10^6 cells/flask. The flask was incubated with F-10 culture solution containing 10% FCS overnight in a CO_2 incubator (5% CO_2 and 100% humidity). The supernatant was removed together with non-adherent cells, and after addition of a fresh culture solution, incubation was continued. On day 6 of the incubation, the cells were confluent and the culture medium was replaced by fresh medium. The next day, the culture solution was discarded, and after addition of 2 ml of anti-mouse red blood cell antibodies (product of Cappel Corporation) which had been diluted 5-fold with RPMI 1640 and 2 ml of a guinea pig complement (product of Kyokuto Seiyaku Co., Ltd.) which had been diluted 2.5-fold with RPMI 1640, the mixture was incubated at 37°C for 20 minutes. After completion of the incubation, the culture was washed twice with 10% F-10 culture solution containing FCS and the nu/nu mouse derived fibroblasts were removed. Subsequently, a F-10 culture solution containing 10% FCS was added and incubation was continued.

(iii) Subculture:

When the initial culture was completely filled with grown cells, it was replaced with F-10 culture solution containing 10% FCS and subculturing was carried out on the following day. After removing the culture solution with a pipette, 2 ml of physiological saline solution containing 0.02% of preheated (37°C) EDTA was added and heated on a hot plate at 37°C for 2 minutes. Thereafter, the cells were detached by pipetting. After addition of 0.5 ml of FCS, the cell suspension was transferred to a 15-ml centrifuge tube and centrifuged at 1,500 rpm (400xg) for 10 minutes to obtain cell pellets. The pellets were suspended in 1 ml of F-10 culture solution and divided into ten portions for subculturing. The same procedures were repeated to perform subculturing at intervals of 4 or 5 days. The reproductive ability of the so obtained cells was examined by the following method. A suspension containing 5×10^4 cells/ml was prepared and twenty 1-ml layers of suspension were transplanted in a plastic dish (35 $mm^{\varnothing}$). During incubation in a CO_2 incubator, the dish was taken out at predetermined intervals and adherent cells were recovered and their number was counted. The results are shown in Figure 4. About 20-24 hours after the cell implantation, cell multiplication started, with a mean duplicating time of about 20 hours.

The so obtained cell line may be used as the cell line capable of producing the G-CSF of the present invention, which is hereunder described by Examples with reference to I-315. It should however, be understood that the scope of the present invention is by no means limited to I-315. For example a microorganism strain or cell line which has been created by a recombinant DNA method is also applicable to this invention.

Examples

Example 1: Isolation of CSF

[0015] In two incubation flasks (150 cm^2) I-315 cells were grown to confluency. The cells were recovered and suspended in 500 ml of F-10 culture solution containing 10% FCS. The cell suspension was transferred to a 1580 cm^2 glass roller bottle and subjected to rotary incubation at 0.5 rpm. When the inner wall of the bottle was completely recovered with grown cells, the culture solution was replaced by serum-free RPMI 1640. After 4-day incubation, the supernatant of the culture was recovered and mixed with F-10 containing 10% FCS for performing continued incubation. After 3-day incubation, the culture solution was again replaced by serum-free RPMI 1640 and subjected to 4-day incubation, followed by recovery of the supernatant of the culture. By repeating the same procedures, 500-ml of serum-free conditioned medium was obtained per bottle every week. This method enables a fairly prolonged cell maintenance and recovery of the conditioned medium.

[0016] To a single batch consisting of 5,000 ml of the recovered conditioned medium, Tween 20® was added at a concentration of 0.01% and the mixture was concentrated about 1000-fold by ultrafiltration using Hollow Fiber DC-4 an Amicon PM-10 (product of Amicon Corporation). The concentrated conditioned medium was purified by the following sequence of steps.

(i) A porton (5 ml) of the concentrate was subjected to gel filtration on a Ultrogel® AcA 54 column (4.6 cm diameter and 90 cm long, product of LKB Corporation) at a flow rate of about 50 ml/hr using 0.01 M Tris-HCl buffer (pH:7.4) containing 0.15 M NaCl and 0.01% Tween® 20 (product of Nakai Kagaku K.K.). The column had been calibrated with bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000) and cytochrome C (mol. wt. 12,400). After the gel filtration, a 0.1-ml portion was sampled from each of the fractions and diluted 10-fold. The activity of each fraction was checked by the "method (b) for determination of CSA". Fractions having V_e=400-700 ml showed macrophage-dominant CSA while fractions having V_e-800-1,200 ml exhibited granulocyte-dominant CSA. There-

fore, the fractions of the second group were combined and concentrated to a volume of about 5 ml by ultrafiltration using PM-10® (product of Amicon Corporation).

(ii) To the concentrated-fractions, a 0.1% aqueous solution of trifluoroacetic acid containing 30% of n-propanol (amino acid sequencing grade, product of Tokyo Kasei K. K.), was added and the mixture was left to stand in ice for about 15 minutes. Thereafter, the mixture was centrifuged at 15,000 rpm (27,000xg) for 10 minutes to remove the precipitate. Then, the supernatant was passed through a micro Bondapak® C18 column (semi-preparatory column produced by Waters Associates, Inc.) that had been equilibrated with an aqueous solution containing n-propanol of the amino acid sequencing grade and trifluoroacetic acid. The column was developed by linear gradient elution with 30-60% of n-propanol containing 0.1% of trifluoroacetic acid. A high-performance liquid chromatography apparatus (Model 685-50 of Hitachi, Ltd.) together with a detector (Model 638-41 of Hitachi, Ltd.) was used for the purpose of simultaneous measurement of absorptions at 220 nm and 280 nm. After elution, a 10 µl portion was separated from each of the fractions and diluted 100-fold. The activity of each fraction was checked by the "method (b) for determination of CSA". The fractions eluted with 40% n-propanol were found to have the neutrophile-dominant CSA so these fractions were collected and subjected to high-performance liquid chromatography under the same conditions as were used above. When the fractions were checked for CSA by the same method as above, it was again confirmed that the peaks corresponding to 40% n-propanol had the neutrophilic granulocytes dominant CSA. Therefore, four fractions (4 ml) of such peaks were collected and freeze-dried

(iii) The freeze-dried powder was dissolved in 200 µl of a 40% n-propanol containing 0.1% trifluoroacetic acid, and the solution was subjected to high-performance liquid chromatography on a TSK-G-3000 SW column (2.5 mm×60 cm, product of Toyo Soda Manufacturing Co., Ltd.). Elution was carried out at 0.4 ml/min with 40% n-propanol containing 0.1% trifluoroacetic acid. With the aid of a fraction collector (FRAC-100 of Pharmacia Fine Chemicals), 0.4-ml fractions were collected. The recovered fractions were checked for their CSA by the same method as used above, and fractions having retention times of 37-38 minutes (corresponding to a molecular weight of ca. 20,000) were found to have the neutrophilic granulocytes dominant CSA. Therefore, these fractions were pooled and purified with an analytical micro Bondapak®C 18 column (4.6 mm×30 cm). Thereafter, the main peaks were recovered and freeze-dried.

Example 2: Physicochemical properties of G-CSF

[0017] The physicochemical properties of the G-CSF of the present invention which was prepared in Example 1 were determined by the following analyses and tests.

2.1 Molecular weight

[0018]

(a) The molecular weight of the G-CSF was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic equipment was Model GE-2/4 (Pharmacia Fine Chemicals) and the gel was made up of a polyacrylamide slab gel (T=15% and C=2.6%) measuring 70 mm×70 mm×3 mm, and a concentrating gel (T=3%, C=20%). T% means total amount of acrylamide (am) plus N,N'-methylenebisacrylamide (bis). C% means percent crosslinking which is calculated as (bis/am plus bis)×100. A modified G-CSF sample was prepared by the following procedure: G-CSF was boiled for 3 minutes in a solution containing 2% of sodium dodecylsulfate in 0.64 M 2-mercaptoethanol, and urea was then added to the solution to a final concentration of 4 M. After performing electrophoresis with 2 µg of the sample at 120 volts for 3 hours, the gel was removed and fixed with methanol:acetic acid:water (4:1:5) and stained with Bio-Rad Silver Stain for band detection. Bovine serum albumin (BSA, mol. wt. 67,000), ovalbumin (OVA, mol. wt. 45,000), cytochrome C (Cyt. C, mol. wt. 12,000) and insulin (Ins., mol. wt. of A chain: 3,300, mol. wt. of B chain: 2,400) were used as molecular weight markers after similar treatments. A single band corresponding to a molecular weight of approximately 18,000 was detected. The results of molecular weight measurement are shown in Figure 1.

(b) The molecular weight of the G-CSF was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), but this time the electrophoretic equipment was Protean® (16 cm, Product of Bio-Rad Corporation), using a gel made up of a polyacrylamide slab gel (T=15%, C=2.6%) measuring 140 mm×160 mm×1.5 mm, and a concentrating gel (T=3%, C=20%). A denatured G-CSF sample was prepared by the following procedure: CSF was boiled for 3 minutes in a solution containing 2% of sodium dodecylsulfate in 0.46 M 2-mercaptoethanol. After performing electrophoresis with 4 µg of the sample with a constant current of 30 mA for 4 hours, the gel was removed and stained with 0.25% Coomassie Brilliant Blue R 250 (product of Sigma Chemical Co.) for band detection. The following substances were used as molecular weight markers after similar treatments: phosphorylase[b] (mol. wt. 92,500), bovine serum albumin (BSA, mol. wt. 67,000), ovalbumin (OVA, mol. wt. 45,000), carbonic anhy-

drase (mol. wt. 31,000), soybean trypsin inhibitor (mol. wt. 21,500) and lysozyme (mol. wt. 14,400). A single band corresponding to a molecular weight of approximately 19,000 was detected. The results of molecular weight measurement are shown in Figure 5.

(c) In view of the results (a) and (b), the G-CSF of the present invention is deemed to have a molecular weight of 19,000±1,000 as determined by SDS-PAGE.

2.2 Isoelectric point

[0019] The isoelectric point of the G-CSF of the present invention was determined by a flat bed isoelectric electrophoretic apparatus, FBE-3000 (product of Pharmacia Fine Chemicals). After 2-hr electrophoresis with a constant power of 30 watts (Vmax=2,000 volts) on a polyacrylamide gel (T=5%, C=3%, 115 mm×230 mm) containing Pharmalyte® (pH=4-6.5, Pharmacia Fine Chemicals) and 4M urea, the G-CSF was fixed with 30% methanol/10% trichloracetic acid/35% sulfosalicylic acid, and stained with Coomassie Brilliant Blue R-250. A Low pl kit (pH: 2.5-6.5, product of Pharmacia Fine Chemicals) was used as an isoelectric point marker.

[0020] Observation of band separation in the pH range of 4 to 6.5 gave three distinct bands corresponding to pl=5.73, 6.03 and 6.37, among which the two bands for pl=5.73 and 6.03 were predominant components. The results of measurements are shown in Figure 2, wherein CSF_0, CSF_1 and CSF_2 denote CSFs according to the present invention having different isoelectric points. Isoelectric electrophoresis in the absence of urea produced three bands corresponding to pl=5.52, 5.80 and 6.13.

[0021] Ten measurements of isoelectric point were conducted by the method described above and the results are shown in Table 1, which lists three isoelectric points, A, B and C, differing from each other by about 0.3.

[0022] In order to see whether there was any correlation between the three bands with different isoelectric points and the values of CSA, the G-CSF which was purified with a TSK-G 3000 SW column in Example 1 (iii) was subjected to band separation with a preparative isoelectric electrophoresis apparatus, Model FBE-3000 of Pharmacia Fine Chemicals. The band separating conditions used were as follows. Sample:

[0023] A freeze-dried sample of G-CSF (500 µg) was dissolved in 1 ml of 0.05N phosphoric acid containing 4M urea.

Support:

[0024] To 15 g of Sephadex-IEF (product of Pharmacia Fine Chemicals), 225 ml of twice-distilled water containing 4M urea and 0.1% Tween 20 was added. After addition of 12 ml of Pharmalyte (pH; 4-6.5, product of Pharmacia Fine Chemicals), the mixture was left to stand overnight for swelling. Thereafter, the mixture was thoroughly deaerated in a sucking bottle and poured on a glass plate (230 mm×230 mm) to form a uniform gel layer in a thickness of 5 mm. The gel layer was removed from the plate except for the most uniform portion covering an area of 50 mm×230 mm.

Electrode solutions:

[0025] Electrode strips (6×10 mm, product of Pharmacia Fins Chemicals) were impregnated with 0.1M phosphoric acid (anode) and 0.1 M NaOH (cathode). One strip was placed parallel to one end of the gel, and the other strip was likewise placed parallel to the other end of the gel. The electrodes were connected to a constant power supply ECPS 2000/300, product of Pharmacia Fins Chemicals.

Preliminary electrophoresis:

[0026] 45 minutes at 8 watts.

Addition of sample:

[0027] A gel portion having a width of 1 cm was scraped out at a position 5 cm away from the anode end and replaced in the initial position after mixing it with a sample solution.

Electrophoresis:

[0028] 4 hours at 50 watts furnished from the constant power supply, ECPS 2000/300.

[0029] After completion of the electrophoresis, the gel was taken out of the tank and divided into 26 fractions with a fractionating grid. After measuring the pH of each of the fractions, the gel scraped from each fraction was transferred to a polypropylene mini-column (Muromac® of Muromachi Kagaku K. K.) and subjected to extraction with 4 ml of 4M

guanidine hydrochloride containing 0.1% of trifluoroacetic acid. A portion (5 μl) of each of the extracted fractions was diluted with 2 ml of RPMI 1640 culture medium containing 1% bovine serum albumin and checked for its CSA by the "method (b) for CSA determination". Each of the fractions eluted contained three active peaks which agreed well with the previously mentioned three isoelectric points pI=5.73, 6.03 and 6.37.

[0030] In order to check whether the differences in isoelectric point should be ascribed to the peptide portion of G-CSF or the sugar chain (especially, the number of additions of sialic acid), two G-CSF samples, one treated with neuraminidase and the other untreated, were subjected to electrophoresis. Three bands were observed in the untreated sample but only a single band for pI=6.37 was observed in the neuraminidase-treated sample. Isoelectric electrophoresis was also conducted for a G-CSF sample that was dissolved in an aqueous solution of 6M guanidine hydrochloric acid, followed by pH adjustment to 1.5 with 1N HCl and standing at 80°C for 120 minutes. Band shifts that occurred in this treated sample were the same as those occurring in the neuraminidase-treated sample. The neuraminidase treatment did not affect the CSA. These results seem to suggest that the differences in isoelectric point of G-CSF are probably due to the difference in the number of additions of sialic acid.

2.3 UV absorption

[0031] The sample was checked for its UV absorption with a spectrophotometer, with 0.1% trifluoroacetic acid containing 40% of n-propanol as a reference. As shown in Figure 3, a maximum occurred at 280 nm and a minimum at 250 nm.

2.4 Amino acids in the protein portion

[0032] The sample was hydrolyzed by a conventional method and analyzed for the amino acid composition of the protein portion with an automatic amino acid analyzer, Model 835 of Hitachi, Ltd. The results are shown in Table 2. The hydrolysis was conducted under the following conditions.

(1) 6N HCl, 110°C×24 hr. in vacuo,
(2) 4N methanesulfonic acid+0.2% 3-(2-aminoethyl)indole, 110°C×24, 48 or 72 hr., in vacuo.

[0033] Each of the samples was dissolved in a solution (1.5 ml) containing 40% n-propanol and 0.1% trifluoroacetic acid. Portions (0.1 ml) of the solutions were dried with dry N_2 gas and mixed with reagent (1) or (2) for hydrolysis in fused test tubes in vacuo.

[0034] Each of the "Measured values" in Table 2 was the average of four values, i.e., the 24-hr value for (1) and 24-, 48- and 72-hr values for (2). The amounts of Thr, Ser, 1/2Cys, Met, Val, Ile and Trp were calculated by the following methods (see, Seikagaku Jikken, Koza, Tanpakushitsu Kagaku II, published by Tokyo Kagaku Dojin):

a) Measurements were made of the time-dependent changes of the 24-, 48- and 72-hr values for Thr, Ser, 1/2Cys and Met after hydrolysis with (2) and the data were extrapolated for zero hour.
b) Measurements were made of the 72-hr values for Val and Ile after hydrolysis with (2).
c) Measurements were made of the 24-, 48- and 72-hr values for Trp after hydrolysis with (2), and these values were averaged.

[0035] The values shown in the column of "Predicted Number of Residual Amino Acid Groups" are based on the assumption of the existence of 33 Leu's. Generally, the amino acids that require correction of the type described above are either decomposed partly or considerably during hydrolysis or refractory to hydrolysis. In particular, Pro produces a low color yield. Primarily for these reasons, the actually measured contents (nmol) of amino acids of interest and hence, the calculated number of each of the residues, has a tendency to be lower than the theoretical values (see Seikagaku Jikken Koza, ibid).

TABLE 2

Aminoacids	Measured values (nmol)	Predicted number of amino acid residues (rounded to the parenthesized integrals)
Asp (Asp+Asn)	3.54	4.3 (4)
Thr	4.58	5.5 (6)

TABLE 2 (continued)

Aminoacids	Measured values (nmol)	Predicted number of amino acid residues (rounded to the parenthesized integrals)
Ser	10.64	12.9 (13)
Glu (Glu+Gln)	22.31	27.0 (27)
Pro	8.30	10.1 (10)
Gly	10.60	12.8 (13)
Ala	14.85	18.0 (18)
1/2Cys	2.59	3.1 (3)
Val	6.16	7.5 (7)
Met	2.26	2.7 (3)
Ile	3.29	4.0 (4)
Leu	27.24	33.0 (33)
Tyr	2.60	3.1 (3)
Phe	5.08	6.1 (6)
Lys	3.68	4.5 (4)
His	3.93	4.8 (5)
Trp	1.61	1.9 (2)
Arg	4.29	5.2 (5)
Total (estimated)		(166)
Calculated molecular weight (no sugar counted in 166 residues)		17961

2.5 Temperature stability

[0036] A freeze-dried G-CSF sample (1 mg) was dissolved in 4 ml of 0.1% trifluoroacetic acid containing 40% of n-propanol. A portion (1 ml) of the solution was diluted with 10 ml of 0.01M Tris-HCl buffer (pH: 7.4) containing 1% bovine serum albumin to give a G-CSF concentration of 25 ng/ml. Five more dilutions of the sample were prepared in the same manner as described above, and the total of six dilutions were treated for 40 minutes at varying temperatures, 0, 37, 45, 56, 65 and 100°C. Determination of the residual CSA in each of the samples showed that the G-CSF of the present invention was stable at 0-45°C and became inactivated at 56°C.

2.6 pH stability

[0037] A freeze-dried G-CSF sample (1 mg) was dissolved in 4 ml of 0.1% trifluoroacetic acid containing 40% of n-propanol. Portions (1 ml) of the solution were diluted with 10-ml solutions containing 1% bovine serum albumin which were buffered at pHs of 1, 3, 5, 7, 9, 11 and 13. After adjusting the CSF concentration to 25 ng/ml, the dilutions were left to stand in ice for 24 hours. Thereafter, 2 ml of each dilution was dialyzed against a 0.01M Tris-HCl buffer (pH: 7.4) and the residual CSA was examined. The G-CSF of the present invention was found to be stable over a broad pH range of 1 to 11.

2.7 Stability against enzymes

[0038] A freeze-dried G-CSF sample was dissolved in 0.1% trifluoroacetic acid containing 40% n-propanol and four specimens were prepared by mixing 0.67 µg of the solution with 0.05M Tris-HCl buffer (pH: 8.0) to a total of 1 ml. Another specimen was prepared by mixing 0.67 µg of the trifluoroacetic acid solution with 0.05M acetate buffer (pH: 5.0) to a total of 1 ml. To three of the first four specimens, RNase, trypsin and pronase were added in amounts of 1 µg, whereas the fourth specimen was used as a control. To the fifth specimen, 1 µg of neuraminidase was added. The five specimens were reacted at 37°C for 2 hours. After completion of the reaction, 0.1-ml portions were taken out of the

specimens and diluted with 1 ml of RPMI 1640 culture solution containing 1% bovine serum albumin. CSA inspection showed that the G-CSF of the present invention was not inactivated by RNase or neuraminidase but was inactivated by trypsin and pronase.

2.8 Sugar composition

[0039] A sample (11 nmol) was mixed with 25 nmol of inositol as an internal standard and 500 µl of 1.5NHCl-methanol, and then subjected to reaction at 90°C for 4 hours in a NO_2 purged fused tube. After completion of the reaction, the tube was opened at one end and supplied with silver carbonate (Ag_2CO_3) for neutralization. After addition of acetic anhydride (50 µl) and shaking, the mixture was left to stand in a dark place overnight at room temperature. The upper layer was placed into a sample tube and dried with N_2 gas. On the other hand, the precipitate was washed with freshly added methanol and lightly centrifuged. The upper layer was poured into the sample tube which had been dried with N_2 gas, and dried again. To the dried content, 50 µl of a TMS reagent (5:1:1 mixture of pyridine, hexamethyl disilazane and trimethylchlorosilane) was added and after reaction at 40°C for 20 minutes, the mixture was stored in a deep freezer. The same procedures were followed except that the internal standard was the combination of 25 nmol of inositol and 50 nmol of one other sugar such as galactose (Gal), N-acetylgalactosamine (Gal NAc) and sialic acid.

[0040] The samples prepared were subjected to gas chromatography under the following conditions.

Assay conditions

[0041]

Column: 2% OV-17 Uniport® HP (60-80 mesh 170-250 µ), 3 m, glass
Temperature: elevated to a temperature between 110°C and 250°C at a rate of 4°C/min
Carrier gas pressure: 1.2-1.6 kg/cm^2 N_2 in the initial stage, and 2-2.5 kg/cm^2 N_2 toward the end of assay
Sensitivity: 10^3 megaohm for a range of 0.1-0.4 volts
Pressure: 0.8 kg/cm^2 H_2, and 0.8 kg/cm^2 air
Sample feed: 2.5-3.0 µl

[0042] Analysis showed that the G-CSF of the present invention contained three sugars, galactose, N-acetylgalactosamine and sialic acid.

2.9 Determination of the N-terminal amino acid sequence

[0043] A sample was subjected to Edman's degradation in a gas-phase sequenator (product of Applied Biosystem, Inc.), and the PTH amino acid obtained was assayed by conventional techniques using a high-performance liquid chromatograph (product of Beckman Instruments, Inc.) and an Ultraphere®-ODS column (product of Beckman Instruments, Inc.). The column (5 µm, 4.6 mm$^{\varnothing}$ and 250 mm^{L}) was first equilibrated with a starting buffer (15 mM sodium acetate buffer, pH=4.5, an aqueous solution containing 40% acetonitrile). Then, a sample dissolved in 20 µl of the starting buffer was subjected to amino acid separation by isocratic elution with the starting buffer. The flow rate was 1.4 ml/min and the column temperature was held at 40°C. Detection of the PTH amino acid was realized by using UV absorption at 269 nm and 320 nm. Samples (2 nmol) of standard PTH amino acid (Sigma Chemical Co.) that had been subjected to amino acid separation in the same system for determination of retention times were used as references against which the retention times for the specimen were compared in order to identify the amino acid sequence. The arrangement of the N-terminal 21 amino acids was as follows:

(10) (20)
H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-X-Leu-Glu-X-Val-

wherein X represents a naturally occurring unidentified amino acid residue.

Example 3: Determination of specific activity

[0044] The specific activity of the G-CSF of Example 1 for human bone marrow cells was determined according to "method (a) for CSA determination". The specific activity was 3.94×10^7 U/mg or higher.

Example 4

[0045] The freeze-dried powder of G-CSF prepared in Example 1 was separated into individual CSF components in terms of differences in isoelectric point as determined by preparative isoelectric electrophoresis under the following conditions.

Equipment:

[0046] FBE-3000 (product of Pharmacia Fine Chemicals)

Sample:

[0047] 10 mg of the freeze-dried powder was dissolved in 2 ml of 0.05N phosphoric acid containing 4M urea.

Support:

[0048] To 15 g of Sephadex-IEF (product of Pharmacia Fine Chemicals), 225 ml of twice-distilled water containing 4M urea and 0.1% Tween 20 was added. After addition of 12 ml of Pharmalyte (pH: 4-6.5, product of Pharmacia Fine Chemicals), the mixture was left to stand overnight for swelling.

[0049] Thereafter, the mixture was thoroughly deaerated in a sucking bottle and poured on a glass plate (230 mm×230 mm) to form a uniform layer in a thickness of 5 mm.

Electrode solutions:

[0050] Electrode strips (6×10 mm, product of Pharmacia Fine Chemicals) were impregnated with 0.1M phosphoric acid (anode) and 0.1M NaOH (cathode). One strip was placed parallel to one end of the gel and the other strip was likewise placed parallel to the other end of the gel. The electrodes were connected to a constant power supply ECPS 2000/300, product of Pharmacia Fine Chemicals.

Preliminary electrophoresis:

[0051] 45 minutes at 8 watts

Addition of sample

[0052] A gel portion having a width of 1 cm was scraped out at a position 5 cm away from the anode and replaced in the initial position after mixing it with a sample solution.

Electrophoresis:

[0053] 4 hours at 50 watts furnished from the constant power supply, ECPS 2000/300.

[0054] After completion of the electrophoresis, the gel was taken out of the tank and divided into 26 fractions with a fractionating grid. After measuring the pH of each of the fractions, the gel scraped from each fraction was transferred to a polypropylene mini-column (Muromac of Muromachi Kagaku K.K.) and subjected to extraction with a 0.1% aqueous trifluoroacetic acid solution (10 ml) containing 4M guanidine hydrochloride. A portion (5 µl) of each of the extracted fractions was diluted with 16ml of RPMI 1640 culture solution containing 1% bovine serum albumin and assayed for its CSA according to "method (b) for CSA determination". Each of the fractions exhibited activity peaks in substantial agreement with three different isoelectric point peaks pI=5.73, 6.03 and 6.37.

[0055] The respective active fractions were adsorbed on a micro Bondapak C 18 column (Waters Associates, Inc., the semi-preparatory grade, 8 mm×30 cm), that had been equilibrated with an aqueous solution containing n-propanol and trifluoroacetic acid. The fractions were then eluted with a 0.1% aqueous trifluoroacetic acid solution containing n-propanol having a linear concentration gradient of 30-60%. The fractions eluted with 40% n-propanol were recovered and freeze-dried.

[0056] The amino acids in the recovered fractions and their sequence were examined according to the methods used in Example 2.4 and 2.9. The results were in agreement with those obtained in Example 2.

Example 5

[0057] A portion (10 mg) of the freeze-dried G-CSF powder prepared in Example 1 was dissolved in 2 ml of a mixture (pH: 9.0) of 0.1M sodium carbonate and sodium bicarbonate. The pH of the solution was adjusted to 5.0 with 1N HCl. After addition of 100 μg of neuraminidase, the mixture was reacted at 37°C for 2 hours, and the reaction mixture was adsorbed on a micro Bondapak®C 18 column (Waters Associates, Inc., the semi-preparatory grade, 8 mmx30 cm) that had been equilibrated with an aqueous solution containing n-propanol and trifluoroacetic acid. The adsorbed mixture was eluted with a 0.1% aqueous solution of trifluoroacetic acid containing n-propanol having a linear concentration gradient of 30-60%. The fractions eluted with 40% n-propanol were recovered and freeze-dried. Pan of the dried powder was subjected to analytical isoelectric electrophoresis, producing a single band corresponding to pI=6.37.

Example 6: Colony classification

[0058] Colonies formed in accordance with the "method (a) for CSA determination" were transferred, together with an agar layer, onto a slide glass by the method of Kubota. K. et al. (Exp. Hemat., *8* (1980), 339-344), and dried to prepare a specimen in a film form. This specimen was subjected to colony classification by both esterase double staining and Biebrich scarlet staining in accordance with the method of Konwalinka, G. et al. (Exp. Hemat., *8* (1980), 434-440). Details of the method of Konwalinka, G. et al. are described below.

(1) Fixing solution: buffered formalin/acetone solution (pH: 6.6)

Na_2HPO_4	20 mg
KH_2PO_4	100 mg
H_2O	30 ml
Acetone	45 ml
Formalin	25 ml
Total	100 ml (stored at 4°C)

(2) Non-specific esterase dyeing reaction solution (prepared just before use) composed of a filtered mixture of the following components (A) and (B):

(A)	Phosphate buffer (1/15 mol/1,000 ml, pH: 6.3)	9.5 ml
	Fast Garnet GBC salt (o-aminoazotoluene, diazonium salt)	10 mg
(B)	α-naphthyl butyrate	10 mg
	Ethylene glycol monomethyl ether	0.5 ml

(3) Chloroacetate esterase dyeing reaction solution (prepared just before use) composed of a filtered mixture of the following components (A) and (B):

(A)	Phosphate buffer (1/15 mol/1,000 ml, pH: 7.4)	9.5 ml
	Fast Blue RR salt (diazotized product of 4-benzoyl-amino-2,5-dimethoxyaniline $ZnCl_2$)	5 mg
(B)	Naphthol AS-D chloroacetate	1 mg
	N,N-dimethylformamide	0.5 ml

(4) Biebrich Scarlet dyeing reaction solution composed of a mixture of 2 ml of solution (A) and 98 ml of solution (B):

(A)	Biebrich Scarlet (product of Metheson Coleman & Bell Corporation	5 g
	Dimethylsulfoxide	100 ml
(B)	0.1M Tris-HCl buffer (pH: 7.4)	

[0059] Using the thus prepared fixing solution and reaction solutions, colony staining was effected in the following order

(i) The colonies were fixed for 30 seconds with the fixing solution (1) at 4-10°C, washed with distilled water three times and dried at room temperature for 10-30 minutes.
(ii) The dried colonies were immersed in the reaction solution (2) at room temperature for 20-30 minutes and washed with distilled water three times.
(iii) The colonies were immersed in the reaction solution (3) at room temperature for 15 minutes and washed with distilled water three times.
(iv) The colonies were immersed in the reaction solution (4) at room temperature for 2 hours and washed under flushing water.
(v) The colonies were dried and observed. Cells containing blue granules were classified as neutrophilic granulocytes. Those containing brown granules were classified as granulocyte-macrophages; and those containing red granules were classified as eosinophiles.

[0060] At days 7, 10 and 14 of the incubation, the colonies formed by using the G-CSF of the present invention were entirely composed of chloroacetate esterase positive granulocytes and no other colony types were found.

Advantages of the invention

[0061] From the following results of experiments (1) to (5), the G-CSF of the present invention was found to have been substantially purified: (1) it exhibited a single peak both in reverse-phase and molecular sieve high-performance liquid chromatographic analyses, and the peak was in agreement with the activity peak; (2) the G-CSF gave a single band in SDS-PAGE; (3) the G-CSF was separated into components having three different isoelectric points upon isoelectric electrophoresis, but each component was a single component exhibiting CSA; a sample that had been freed from a terminal sialic acid either by enzymatic or chemical techniques gave a single band in isoelectric electrophoresis; (4) only a single type of PTH amino acid appeared in each of the steps involved in analysis of the seqeuence of 21 amino acid residues from N terminal; and (5) in terms of specific activity, the G-CSF is about 10 times as pure as those CSFs so far reported as being effective in humans.

[0062] Such a highly purified form of G-CSF, which has the ability to promote the differentiation and proliferation of human bone marrow cells to granulocytes, is not to be found in available literature. The G-CSF of the present invention is obtainable from a genetically engineered human G-CSF producing strain. In addition to use as a reagent for clinical testing or research studies, the G-CSF of the present invention may potentially be used as a curative for serious infections diseases so far incurable by antibiotics because it has various capabilities, i.e. promotion of the proliferation of the cells of a transplanted bone marrow, promotion of the restoration of radiation exposed bone marrow tissues, promotion of the restoration of the leukocyte level after application of cancer control agents, and promotion of the differentiation and proliferation of bone marrow cells to granulocytes.

Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, IT, LI, NL, SE

1. Human granulocyte colony stimulating factor (hG-CSF) having a specific activity of at least 3.94×10^7 U/mg in the human bone marrow cell assay and the ability of promoting the differentiation and proliferation of human bone marrow cells to neutrophilic granulocytes but not to granulocyte-macrophages and not to eosinophils in the human have marrow cell assay at days 7, 10 and 14 of the incubation and having the following physicochemical properties:

(i) Molecular weight: 19,000 ± 1,000 as determined by sodium dodecylsulfatepolyacrylamide gel electrophoresis;

(ii) Isoelectric point: having at least one of the three isoelectric points A, B and C, shown in Table 1:

Table 1

Isoelectric point (pI)		
	In the presence of 4M urea	In the absence of urea
A	5.7 ± 0.1	5.5 ± 0.1
B	6.0 ± 0.1	5.8 ± 0.1
C	6.3 ± 0.1	6.1 ± 0.1

(iii) UV absorption: Maximum absorption at 280 nm and minimum absorption at 250 nm;
(iv) The N-terminal 21 amino acids are

```
                       (5)                     (10)
H2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-

                      (17)        (20)
Ser-Phe-Leu-Leu-Lys-X-Leu-Glu-X-Val-,
```

wherein X represents a naturally occurring unidentified amino acid residue.

2. The hG-CSF according to claim 1 wherein the isoelectric point pI is A.

3. The hG-CSF according to claim 1 wherein the isoelectric point pI is B.

4. The hG-CSF according to claim 1 wherein the isoelectric point pI is C.

5. The hG-CSF according to claim 1 wherein the molecule is glycosylated.

6. A method for preparing the hG-CSF according to claim 1, said method comprising culturing a cell line having an hG-CSF producing ability in a serum-free culture solution, subjecting the supernatant of the culture to steps (1) to (3) indicated below, and optionally subjecting the resulting fractions to either step (4) or (5):

step (1) subjecting the supernatant of the culture to gel filtration using a gel having an effective fraction range of 5,000 - 70,000 daltons, and recovering fractions having the neutrophile-dominant colony stimulating activity (CSA);

step (2) adsorbing the recovered fractions in a 0.1% aqueous solution of trifluoroacetic acid containing 30% of n-propanol onto a carrier for reverse-phase high-performance liquid chromatography and performing elution by a linear gradient of 30-60% of n-propanol containing 0.1% of trifluoroacetic acid so as to recover fractions having the neurophile-dominant CSA;

step (3) subjecting the so recovered fractions to high-performance molecular sieve chromatography so as to recover fractions having the neutrophile-dominant CSA;

step (4) subjecting the so recovered fractions to isoelectric point electrophoresis so as to recover fractions having the neurophile-dominant CSA; or

step (5) subjecting the fractions recovered in step (3) to the step of removing sialic acid so as to recover fractions having the neutrophile-dominant CSA.

7. The method according to claim 6 wherein the cell line having the hG-CSF producing ability is C.N.C.M. I-315.

8. Pharmaceutical composition comprising the hG-CSF according to claims 1 to 5.

9. Pharmaceutical composition for stimulating the differentiation and proliferation of bone marrow cells to neutrophilic granulocytes comprising an effective amount of the hG-CSF according to claims 1 to 5.

10. Pharmaceutical composition for assisting in curing infections diseases comprising an effective amount of the hG-CSF according to claims 1 to 5.

Claims for the following Contracting State : AT

1. A method for obtaining a human granulocyte colony stimulating factor (hG-CSF) having a specific activity of at least 3.94×10^7 U/mg in the human bone marrow cell assay and the ability of promoting the differentiation and proliferation of human bone marrow cells to neutrophilic granulocytes but not to granulocyte-macrophages and not to eosinophils in the human bone marrow cell assay at days 7, 10 and 14 of the incubation and having the following physicochemical properties:

   (v) Molecular weight: 19,000 ± 1,000 as determined by sodium dodecylsulfatepolyacrylamide gel electrophoresis;
   (vi) Isoelectric point: having at least one of the three isoelectric points A, B and C, shown in Table 1:

Table 1

Isoelectric point (pI)		
	In the presence of 4M urea	In the absence of urea
A	5.7 ± 0.1	5.5 ± 0.1
B	6.0 ± 0.1	5.8 ± 0.1
C	6.3 ± 0.1	6.1 ± 0.1

   (vii) UV absorption: Maximum absorption at 280 nm and minimum absorption at 250 nm;
   (viii) The N-terminal 21 amino acids are

```
                     (5)                        (10)
H2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-

                     (17)         (20)
Ser-Phe-Leu-Leu-Lys-X-Leu-Glu-X-Val-,
```

   wherein X represents a naturally occurring unidentified amino acid residue, said method comprising culturing a cell line having an hG-CSF producing ability in a serum-free culture solution, subjecting the supernatant of the culture to steps (1) to (3) indicated below, and optionally subjecting the resulting fractions to either step (4) or (5):

   step (1) subjecting the supernatant of the culture to gel filtration using a gel having an effective fraction range of 5,000 - 70,000 daltons, and recovering fractions having the neutrophile-dominant colony stimulating activity (CSA);
   step (2) adsorbing the recovered fractions in a 0.1% aqueous solution of trifluoroacetic acid containing 30% of n-propanol onto a carrier for reverse-phase high-performance liquid chromatography and performing elution by a linear gradient of 30-60% of n-propanol containing 0.1% of trifluoroacetic acid so as to recover fractions having the neurophile-dominant CSA;
   step (3) subjecting the so recovered fractions to high-performance molecular sieve chromatography so as to recover fractions having the neutrophile-dominant CSA;
   step (4) subjecting the so recovered fractions to isoelectric point electrophoresis so as to recover fractions having the neurophile-dominant CSA; or
   step (5) subjecting the fractions recovered in step (3) to the step of removing sialic acid so as to recover fractions having the neutrophile-dominant CSA.

2. The method according to claim 1, wherein the hG-CSF obtained is glycosylated.

3. The method according to claim 1, wherein the cell line having the hG-CSF producing ability is C.N.C.M. I-315.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, IT, LI, NL, SE

1. Menschlicher Granulozytenkolonie-stimulierender Faktor (hG-CSF), der eine spezifische Aktivität von mindestens 3,94 x 10^7 U/mg in dem Test an menschlichen Knochenmarkzellen und die Fähigkeit aufweist, in dem Test an menschlichen Knochenmarkzellen nach 7-, 10- und 14-tägiger Inkubation die Differenzierung und Proliferation menschlicher Knochenmarkzellen zu neutrophilen Granulozyten, jedoch nicht zu Granulozyten-Makrophagen und nicht zu Eosinophilen, zu fördern, und der die folgenden physikalisch-chemischen Eigenschaften besitzt:

   (i) Molekulargewicht: 19.000±1.000, bestimmt durch Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese;
   (ii) isoelektrischer Punkt: mindestens einer der drei isoelektrischen Punkte A, B und C gemäß Tabelle 1:

TABELLE 1

Isoelektrischer Punkt (pI)		
	In Gegenwart von 4M Harnstoff	In Abwesenheit von Harnstoff
A	5,7±0,1	5,5±0,1
B	6,0±0,1	5,8±0,1
C	6,3±0,1	6,1±0,1

   (iii) UV-Absorption: Absorptionsmaximum bei 280 nm und Absorptions-minimum bei 250 nm;
   (iv) die N-terminalen 21 Aminosäuren sind

(5) (10) (17)
H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-X-

(20)
Leu-Glu-X-Val-,

   wobei X einen natürlich vorkommenden, nicht-identifizierten Aminosäurerest darstellt.

2. hG-CSF nach Anspruch 1 mit dem isoelektrischen Punkt pI=A.

3. hG-CSF nach Anspruch 1 mit dem isoelektrischen Punkt pI=B.

4. hG-CSF nach Anspruch 1 mit dem isoelektrischen Punkt pI=C.

5. hG-CSF nach Anspruch 1, wobei das Molekül glykosyliert ist.

6. Verfahren zur Herstellung des hG-CSF nach Anspruch 1, umfassend das Züchten einer zur Produktion von hG-CSF fähigen Zellinie in einer Serumfreien Kulturlösung, das Durchführen der nachstehend angegebenen Schritte (1)-(3) mit dem Kulturüberstand und gegebenenfalls das Durchführen entweder des Schritts (4) oder (5) mit den erhaltenen Fraktionen:

Schrill (1)	Gelfiltration des Kulturüberstands, wobei ein Gel verwendet wird, das einen wirksamen Fraktionsbereich von 5.000 bis 70.000 Dalton aufweist, und Isolierung der Fraktionen mit neutrophildominanter koloniestimulierender Aktivität (CSA);
Schritt (2)	Adsorption der isolierten Fraktionen in einer 0.1%-igen wäßrigen 30% n-Propanol enthaltenden Lösung von Trifluoressigsäure an einen Träger für Reverse-Phase-Hochleistungs-Flüssigkeitschromatographie und Elution mittels eines linearen Gradienten von 0.1% Trifluoressigsäure enthaltendem 30-60% n-Propanol zur Gewinnung von Fraktionen mit neutrophil-dominanter CSA;
Schritt (3)	Durchführung einer Hochleistungs-Molekularsieb-Chromatographie mit den gewonnenen Fraktionen zur Isolierung von Fraktionen mit neutrophil-dominanter CSA;
Schrill (4)	Durchführung einer isoelektrischen Punkt-Elektrophorese mit den isolierten Fraktionen zur Gewinnung von Fraktionen mit neutrophil-dominanter CSA; oder
Schritt (5)	Behandlung der in Schritt (3) isolierten Fraktionen zur Entfernung von Sialinsäure zur Gewinnung von Fraktionen mit neutrophil-dominanter CSA.

7. Verfahren nach Anspruch 6, wobei die zur Produktion von hG-CSF fähige Zellinie C.N.C.M. I-315 ist.

8. Arzneimittel, umfassend den hG-CSF nach den Ansprüchen 1-5.

9. Arzneimittel zur Stimulierung der Differenzierung und Proliferation von Knochenmarkzellen zu neutrophilen Granulozyten, umfassend eine wirksame Menge des hG-CSF nach den Ansprüchen 1-5.

10. Arzneimittel zur unterstützenden Behandlung von infektiösen Erkrankungen, umfassend eine wirksame Menge des hG-CSF nach den Ansprüchen 1-5.

Patentansprüche für folgenden Vertragsstaat : AT

1. Verfahren zur Gewinnung eines menschlichen Granulozytenkolonlestimulierenden Faktors (hG-CSF), der eine spezifische Aktivität von mindestens $3{,}94 \times 10^7$ U/mg in dem Test an menschlichen Knochenmarkzellen und die Fähigkeit aufweist, in dem Test an menschlichen Knochenmarkzellen nach 7-, 10- und 14-tägiger Inkubation die Differenzierung und Proliferation menschlicher Knochenmarkzellen zu neutrophilen Granulozyten, jedoch nicht zu Granulozyten-Makrophagen und nicht zu Eosinophilen, zu fördern, und der die folgenden physikalisch-chemischen Eigenschaften besitzt:

   (i) Molekulargewicht: 19.000±1.000, bestimmt durch Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese;
   (ii) isoelektrischer Punkt: mindestens einer der drei isoelektrischen Punkte A, B und C gemäß Tabelle 1:

TABELLE 1

Isoelektrischer Punkt (pI)		
	In Gegenwart von 4M Harnstoff	In Abwesenheit von Harnstoff
A	5,7±0,1	5,5±0,1
B	6,0±0,1	5,8±0,1
C	6,3±0,1	6,1±0,1

(iii) UV-Absorption: Absorptionsmaximum bei 280 nm und Absorptionsminimum bei 250 nm;
(iv) die N-terminalen 21 Aminosäuren sind

(5) (10) (17)
H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-X-

(20)
Leu-Glu-X-Val-,

wobei X einen natürlich vorkommenden, nicht-identifizierten Aminosäurerest darstellt,
wobei das Verfahren das Züchten einer zur Produktion von hG-CSF fähigen Zellinie in einer Serum-freien Kulturlösung, das Durchführen der nachstehend angegebenen Schritte (1)-(3) mit dem Kulturüberstand und gegebenenfalls das Durchführen entweder des Schritts (4) oder (5) mit den erhaltenen Fraktionen:

Schritt (1) Gelfiltration des Kulturüberstands, wobei ein Gel verwendet wird, das einen wirksamen Fraktionsbereich von 5.000 bis 70.000 Dalton aufweist, und Isolierung der Fraktionen mit neutrophil-dominanter koloniestimulierender Aktivität (CSA);

Schritt (2) Adsorption der isolierten Fraktionen in einer 0.1%-igen wäßrigen 30% n-Propanol enthaltenden Lösung von Trifluoressigsäure an einen Träger für Reverse-Phase-Hochleistungs-Flüssigkeitschromatographie und Elution mittels eines linearen Gradienten von 0.1% Trifluoressigsäure enthaltendem 30-60% n-Propanol zur Gewinnung von Fraktionen mit neutrophil-dominanter CSA;

Schritt (3) Durchführung einer Hochleistungs-Molekularsieb-Chromatographie mit den gewonnenen Fraktionen zur Isolierung von Fraktionen mit neutrophil-dominanter CSA;

Schritt (4) Durchführung einer isoelektrischen Punkt-Elektrophorese mit den isolierten Fraktionen zur Gewinnung von Fraktionen mit neutrophil-dominanter CSA; oder

Schritt (5) Behandlung der in Schritt (3) isolierten Fraktionen zur Entfernung von Sialinsäure zur Gewinnung von Fraktionen mit neutrophil-dominanter CSA.

2. Verfahren nach Anspruch 1, wobei der gewonnene hG-CSF glykosyliert ist.

3. Verfahren nach Anspruch 1, wobei die zur Produktion von hG-CSF fähige Zellinie C.N.C.M. I-315 ist.

Revendications

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, IT, LI, NL, SE

1. Facteur humain de stimulation de colonie de granulocytes (hG-CSF) présentant une activité spécifique d'au moins $3{,}94 \times 10^7$ U/mg dans un test sur cellules de moelle osseuse humaine et ayant l'aptitude d'activer la différenciation et la prolifération des cellules de moelle osseuse humaine en granulocytes neutrophiles mais pas en granulocytes macrophages ni en éosinophiles dans un test sur cellules de moelle osseuse humaine aux jours 7, 10 et 14 de l'incubation, et ayant les propriétés physico-chimiques suivantes :

(i) Poids moléculaire :
19 000 ± 1 000, déterminé par électrophorèse sur gel de dodécylsufate de sodium et de polyacrylamide ;

(ii) Point isoélectrique :
possédant au moins un des trois points isoélectriques A, B et C mentionnés dans le tableau 1 :

Tableau 1

	Point isoélectrique (pI)	
	en présence d'urée 4M	en l'absence d'urée
A	5,7 ± 0,1	5,5 ± 0,1
B	6,0 ± 0,1	5,8 ± 0,1
C	6,3 ± 0,1	6,1 ± 0,1

(iii) Absorption UV : absorption maximum à 280 nm et absorption minimum à 250 nm ;

(iv) la séquence de 21 amino-acides N-terminale est:

(5) (10) (17) (20)

H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-X-Leu-Glu-X-Val- ;

dans laquelle X représente un groupe amino acide naturel non identifié.

2. hG-CSF selon la revendication 1, dans lequel le point isoélectrique pI est A.

3. hG-CSF selon la revendication 1, dans lequel le point isoélectrique pI est B.

4. hG-CSF selon la revendication 1, dans lequel le point isoélectrique pI est C.

5. hG-CSF selon la revendication 1, dans lequel la molécule est glycosylée.

6. Procédé de préparation du hG-CSF selon la revendication 1, dans lequel on cultive une lignée de cellules ayant l'aptitude de produire un hG-CSF dans une solution de culture exempte de sérum, on soumet le surnageant de la culture aux étapes (1) à (3) mentionnées ci-dessous, et on soumet éventuellement les fractions résultantes soit à l'étape (4) soit à l'étape (5) :

   étape (1) : on soumet le surnageant de la culture à une filtration sur gel en utilisant un gel ayant une plage de fractionnement efficace allant de 5 000 à 70 000 daltons et on récupère les fractions possédant l'activité de stimulation de colonie (CSA) à dominante neutrophile ;
   étape (2) : on adsorbe les fractions récupérées dans une solution aqueuse à 0,1 % d'acide trifluoroacétique contenant 30 % de n-propanol sur un support pour une chromatographie liquide haute performance à phase inverse et on réalise l'élution avec un gradient linéaire de 30-60 % de n-propanol contenant 0,1 % d'acide trifluoroacétique de façon à récupérer les fractions ayant la CSA à dominante neutrophile ;
   étape (3) : on soumet les fractions ainsi récupérées à une chromatographie haute performance sur tamis moléculaire de façon à récupérer les fractions ayant la CSA à dominante neutrophile ;
   étape (4) : on soumet les fractions ainsi récupérées à une électrophorèse isoélectrique de façon à récupérer les fractions ayant la CSA à dominante neutrophile ; ou
   étape (5) : on soumet les fractions récupérées dans l'étape (3) à l'étape d'élimination de l'acide sialique de façon à récupérer les fractions ayant la CSA à dominante neutrophile.

7. Procédé suivant la revendication 6, dans lequel la lignée de cellules ayant l'aptitude de produire le hG-CSF est la lignée CNCM n° de dépôt I-315.

8. Composition pharmaceutique comprenant le hG-CSF selon les revendications 1 à 5.

9. Composition pharmaceutique pour stimuler la différenciation et la prolifération des cellules de moelle osseuse en

granulocytes neutrophiles comprenant une quantité efficace de hG-CSF selon les revendications 1 à 5.

10. Composition pharmaceutique pour l'aide au traitement de maladies infectieuses comprenant une quantité efficace de hG-CSF selon les revendications 1 à 5.

Revendications pour l'Etat contractant suivant : AT

1. Procédé d'obtention d'un facteur humain de stimulation de colonie de granulocytes (hG-CSF) présentant une activité spécifique d'au moins $3,94 \times 10^7$ U/mg dans un test sur cellules de moelle osseuse humaine et ayant l'aptitude d'activer la différenciation et la prolifération des cellules de moelle osseuse humaine en granulocytes neutrophiles mais pas en granulocytes macrophages ni en éosinophiles dans un test sur cellules de moelle osseuse humaine aux jours 7, 10 et 14 de l'incubation, et ayant les propriétés physico-chimiques suivantes :

   (i) Poids moléculaire :
   19 000 ± 1 000, déterminé par électrophorèse sur gel de dodécylsufate de sodium et de polyacrylamide ;

   (ii) Point isoélectrique :
   possédant au moins un des trois points isoélectriques A, B et C mentionnés dans le tableau 1 :

Tableau 1

	Point isoélectrique (pl)	
	en présence d'urée 4M	en l'absence d'urée
A	5,7 ± 0,1	5,5 ± 0,1
B	6,0 ± 0,1	5,8 ± 0,1
C	6,3 ± 0,1	6,1 ± 0,1

   (iii) Absorption UV:
   absorption maximum à 280 nm et absorption minimum à 250 nm ;

   (iv) la séquence de 21 amino-acides N-terminale est :

(5) (10) (17) (20)

H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-X-Leu-Glu-X-Val- ;

   dans laquelle X représente un groupe amino acide naturel non identifié ;
   dans lequel on cultive une lignée de cellules ayant l'aptitude de produire un hG-CSF dans une solution de culture exempte de sérum, on soumet le surnageant de la culture aux étapes (1) à (3) mentionnées ci-dessous, et on soumet éventuellement les fractions résultantes soit à l'étape (4) soit à l'étape (5) :

      étape (1) : on soumet le surnageant de la culture à une filtration sur gel en utilisant un gel ayant une plage de fractionnement efficace allant de 5 000 à 70 000 daltons et on récupère les fractions possédant l'activité de stimulation de colonie (CSA) à dominante neutrophile ;
      étape (2) : on adsorbe les fractions récupérées dans une solution aqueuse à 0,1 % d'acide trifluoroacétique contenant 30 % de n-propanol sur un support pour une chromatographie liquide haute performance à phase inverse et on réalise l'élution avec un gradient linéaire de 30-60 % de n-propanol contenant 0,1 % d'acide trifluoroacétique de façon à récupérer les fractions ayant la CSA à dominante neutrophile ;
      étape (3) : on soumet les fractions ainsi récupérées à une chromatographie haute performance sur tamis moléculaire de façon à récupérer les fractions ayant la CSA à dominante neutrophile ;
      étape (4) : on soumet les fractions ainsi récupérées à une électrophorèse isoélectrique de façon à récupérer les fractions ayant la CSA à dominante neutrophile ; ou
      étape (5) : on soumet les fractions récupérées dans l'étape (3) à l'étape d'élimination de l'acide sialique de

façon à récupérer les fractions ayant la CSA à dominante neutrophile.

2. Procédé selon la revendication 1, dans lequel le hCSF obtenu est glycosylé.

3. Procédé selon la revendication 1, dans lequel la lignée de cellules ayant l'aptitude de produire le hG-CSF est la lignée CNCM n° de dépôt I-315.

Fig. 1

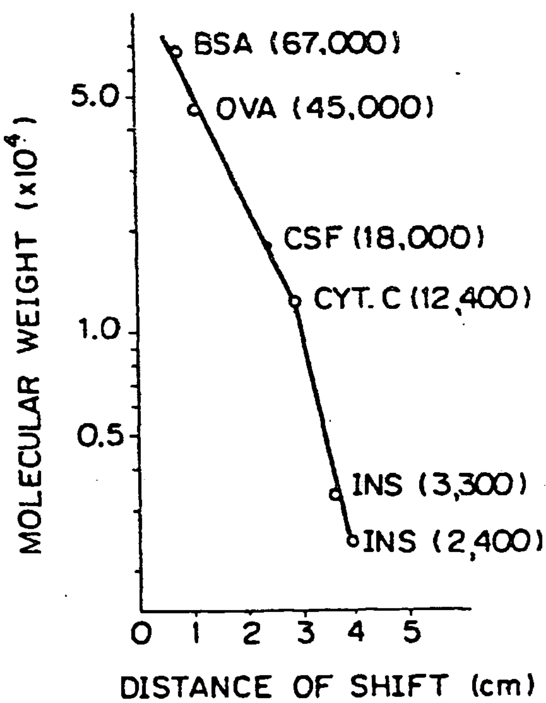

BSA (67,000)
OVA (45,000)
CSF (18,000)
CYT. C (12,400)
INS (3,300)
INS (2,400)
5.0
1.0
0.5
MOLECULAR WEIGHT (x10⁴)
0
1
2
3
4
5
DISTANCE OF SHIFT (cm)

Fig. 2

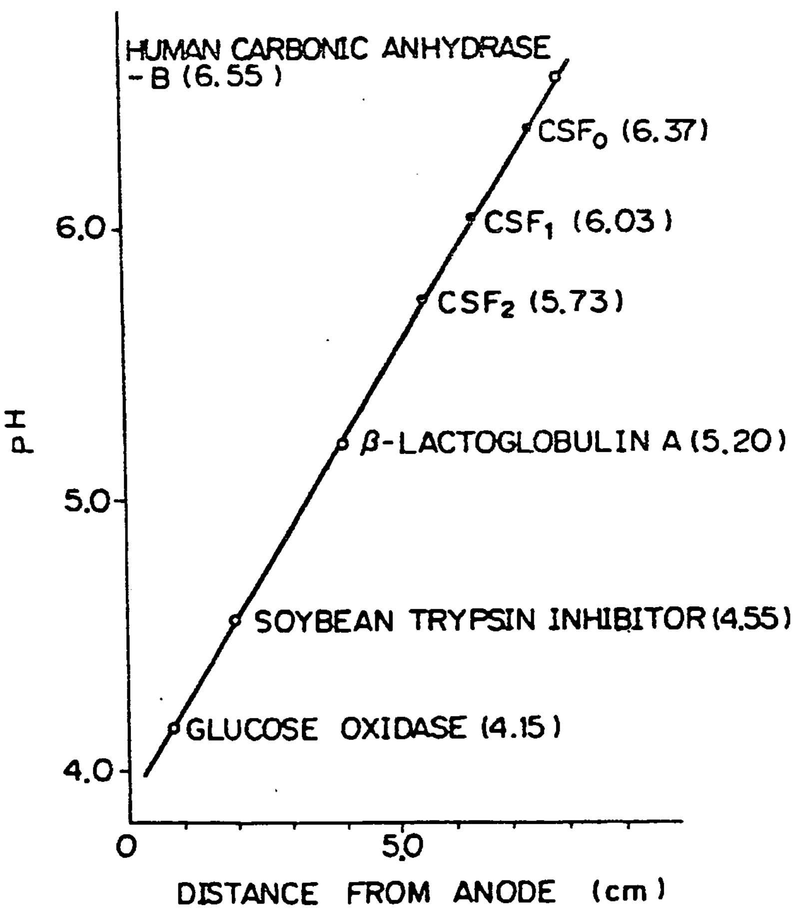

HUMAN CARBONIC ANHYDRASE
-B (6.55)
CSF0 (6.37)
CSF1 (6.03)
CSF2 (5.73)
β-LACTOGLOBULIN A (5.20)
SOYBEAN TRYPSIN INHIBITOR (4.55)
GLUCOSE OXIDASE (4.15)
pH
6.0
5.0
4.0
0
5.0
DISTANCE FROM ANODE (cm)

Fig. 3

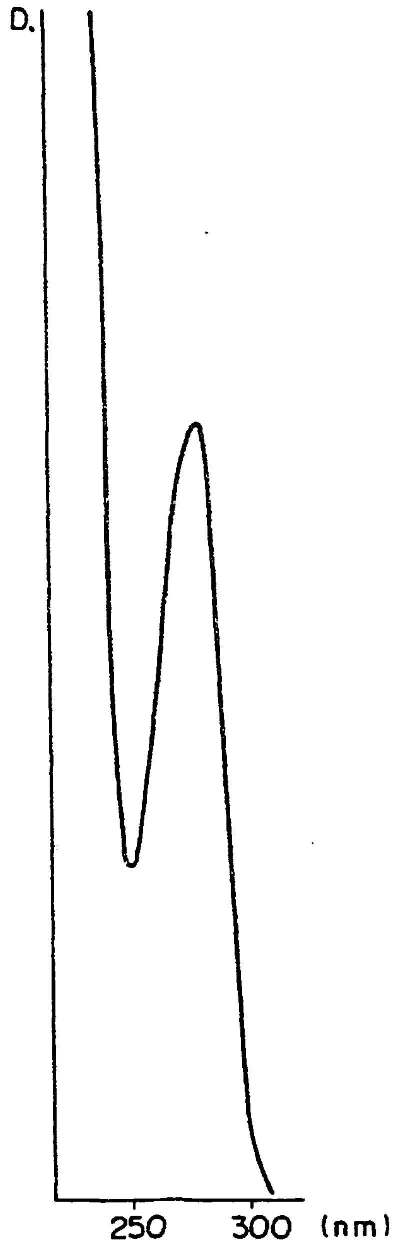

D.
250
300
(nm)

Fig. 4

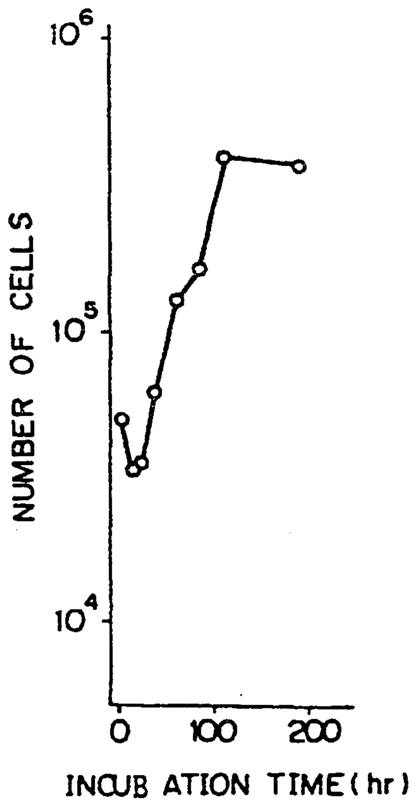
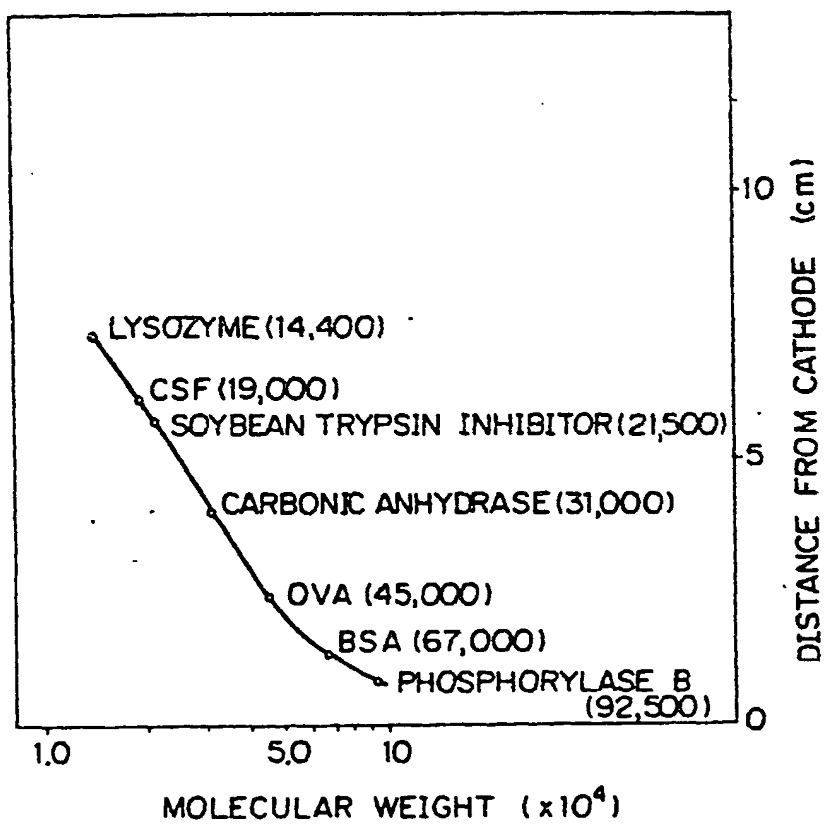

10^6
10^5
10^4
NUMBER OF CELLS
0
100
200
INCUB ATION TIME(hr)

Fig. 5


LYSOZYME (14,400)
CSF (19,000)
SOYBEAN TRYPSIN INHIBITOR (21,500)
CARBONIC ANHYDRASE (31,000)
OVA (45,000)
BSA (67,000)
PHOSPHORYLASE B (92,500)
10
5
0
DISTANCE FROM CATHODE (cm)
1.0
5.0
10
MOLECULAR WEIGHT (x10^4)